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RESEARCH PAPER

Delayed maturation of nodules reduces symbiotic effectiveness of the *Lotus japonicus–Rhizobium* sp. NGR234 interaction

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Abstract

Lotus japonicus, a model legume, develops an efficient, nitrogen-fixing symbiosis with _Mesorhizobium loti_ that promotes plant growth. _Lotus japonicus_ also forms functional nodules with _Rhizobium_ sp. NGR234 and _R. etli_. Yet, in a plant defence-like reaction, nodules induced by _R. etli_ quickly degenerate, thus limiting plant growth. In contrast, nodules containing NGR234 are long-lasting. It was found that NGR234 initiates nodule formation in a similar way to _M. loti_ MAFF303099, but that the nodules which develop on eleven _L. japonicus_ ecotypes are less efficient in fixing nitrogen. Detailed examination of nodulation of _L. japonicus_ cultivar MG-20 revealed that symbiosomes formed four weeks after inoculation by NGR234 are enlarged in comparison with MAFF303099 and contain multiple bacteroids. Nevertheless, nodules formed by NGR234 fix sufficient nitrogen to avoid rejection by the plant. With time, these nodules develop into fully efficient organs containing bacteroids tightly enclosed in symbiosome membranes, just like those formed by _M. loti_ MAFF303099. This work demonstrates the usefulness of using the well-characterized micro-symbiont NGR234 to study symbiotic signal exchange in the later stages of rhizobia–legume symbioses, especially given the large range of bacterial (NGR234) and plant (_L. japonicus_) mutants that are available.

Key words: Infection, *Mesorhizobium*, nodulation, persistence, rhizobia, senescence, symbiosis, symbiosomes.

Introduction

An intense exchange of molecular signals between compatible legumes and rhizobia leads to the establishment of symbioses, in which atmospheric nitrogen is reduced to ammonia that is incorporated into the plants. In return, fixed carbon is supplied to the rhizobia. Nod-factors are the key bacterial signals that allow rhizobia to enter root hairs (Relić *et al.*, 1993, 1994*a*, *b*; D'Haeze *et al.*, 1998) and re-programme root cell development towards new organs called nodules to accommodate the rhizobia (Broughton *et al.*, 2000). As they penetrate the root, rhizobia are internalized into cortical cells where they become enclosed in a plant-derived membrane, giving rise to symbiosomes (Verma and Hong, 1996; Jones *et al.*, 2007). Subsequent differentiation of the rhizobia into nitrogen-fixing bacteroids concludes nodule development. Thereafter, a second phase of symbiotic interaction begins that is characterized by a long-lasting intracellular existence of the rhizobia, which requires a continuous metabolite exchange between the two partners (Lodwig and Poole, 2003).

Failure to establish successful symbioses, or the breakdown of symbioses, occurs between incompatible legumes and rhizobia. Many examples of rhizobia that attempt to invade legumes exist, but nodule development is often arrested at various levels because one or more of the appropriate symbiotic signals is missing. Bacterial persistence within the host, the second phase of symbiotic interactions, has,

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however, not been as well studied. Indeed, rhizobial mutants unable to fix nitrogen develop normal nodules. Some days later, however, the symbiosomes show signs of senescence and the nodules degenerate (Hahn and Studer, 1986; Hirsch and Smith, 1987). Additionally, Kiers *et al.* (2003) have shown that inefficiency on the part of the micro-symbiont leads the plant host to restrict the symbiotic interaction. Taken together, these data illustrate that nitrogen fixation by rhizobia is necessary for intracellular persistence within plant tissues.

Lotus japonicus is an important model legume that is widely used to study symbiotic interactions with micro-organisms (Udvardi *et al.*, 2005), and significant advances have been made in the understanding of Nod-factor perception by *L. japonicus* (Kistner *et al.*, 2005; Radutoiu *et al.*, 2007, and references therein). In the wild, *L. japonicus* is nodulated by strains of *Mesorhizobium loti*. Interestingly, *M. loti* and *Rhizobium etli* strain CE3 secrete identical Nod-factors (Cardenas *et al.*, 1995; Lopez-Lara *et al.*, 1995), and *R. etli* CE3 induces effective nodules on *L. japonicus* (Banba *et al.*, 2001). About 3 weeks post-inoculation (wpi) nitrogen fixation suddenly stops and the nodules begin to senesce prematurely. It was suggested that delayed recognition of *R. etli* CE3 as an 'irregular' micro-symbiont of *Lotus* occurs, and that this triggers some form of plant defence (Banba *et al.*, 2001). Such transient symbiotic interactions mean that nitrogen fixation is not the only requirement for rhizobial persistence in nodules. Furthermore, they point to the presence of additional plant check-points at later stages of the symbiosis and concomitant signals dedicated to persistence within the plant cells. Perhaps these signals employ mechanisms similar to those used by some pathogenic bacteria to maintain long-lasting (chronic) intracellular infections in their eukaryotic hosts (LeVier *et al.*, 2000; Rhen *et al.*, 2003; Monack *et al.*, 2004).

Rhizobium species NGR234 (hereafter NGR234) is an exceptionally broad host-range micro-symbiont (Pueppke and Broughton, 1999) that utilizes a large diversity of well-characterized symbiotic signals (Perret *et al.*, 2000). Amongst these are a complex mixture of Nod-factors, some of which are identical to those synthesized by *M. loti* and *R. etli* CE3 (Price *et al.*, 1992). As a probable consequence, NGR234 induces (4–6 wpi) effective nodules on *L. japonicus* (Hussain *et al.*, 1999; Pueppke and Broughton, 1999). Signs of premature senescence have not been reported. A subsequent study showed that nodules induced by NGR234 were still effective 12 wpi (Müller *et al.*, 2001) even though NGR234 is not a 'regular' micro-symbiont of *L. japonicus*. This apparent discrepancy was investigated by comparing nodulation of *L. japonicus* by NGR234 and *M. loti* MAFF303099 (hereafter MAFF303099) at different stages of the symbiotic interaction.

Materials and methods

Plant material

Lotus seeds were scarified with glass paper, incubated in 100% ethanol for 10 min, and surface sterilized for 1 h in 0.35% Chlorox at 27 °C with gentle agitation. Seeds were washed three times with sterile double-distilled water (ddH_2O) and incubated overnight in ddH_2O at 4 °C. The next day, they were rinsed three times with ddH_2O and placed in sterile Magenta™ jars (Magenta Corp., Chicago, IL, USA) containing clay beads of 2–4 mm diameter (InterHydro AG, Allmendingen, Switzerland) and 30 ml of four-times diluted B&D nutrient solution (Broughton and Dilworth, 1971). Seeds were allowed to germinate for 4 d in the dark at room temperature. Three fully germinated seedlings were planted into clay beads mixed with 75 ml of diluted B&D in a Magenta jar, covered with 1 cm of quartz sand, and closed by fixing a second (inverted) jar above. Nodulation tests lasting for longer (e.g. 8 wpi) were performed as described by Lewin *et al.* (1990) using diluted B&D. In both cases, each jar was inoculated with 10^6 bacteria re-suspended in 1 ml of 10 mM $MgSO_4$ (330 µl per plant) 4 d after plantlet transfer into Magenta jars. Light intensity was kept at 550 µE m^2 s^{-1} for 16 h d^{-1} at 20 °C.

Microbiology

Rhizobial strains and plasmids are listed in Supplementary Table S1 available at *JXB* online. Rhizobia were raised at 28 °C in TY medium (Beringer, 1974). When appropriate, rifampicin, phosphomycin, and tetracycline were added at concentrations of 100, 100, and 15 mg ml^{-1}, respectively.

Electron and light microscopy

Nodules were harvested and cut into two equal parts along a transversal plane. Half of the nodules were fixed overnight at 4 °C with 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7). Samples were washed four times (15 min each time) in Na-cacodylate buffer and post-fixed with 1% (w/v) OsO_4 in Na-cacodylate buffer for 2 h at 4°C. Then, they were rinsed in Na-cacodylate buffer, dehydrated in an ethanol series, and embedded in Epon 812 (Fluka, Buchs, Switzerland). Semi-thin sections (1 µm) were stained with methylene blue and basic fuchsin before observation with a Leica DMIRE 2 microscope (Leica Microsystems GmbH, Wetzlar, Germany). Ultra-thin (60 nm) sections were stained with 2% (w/v) aqueous uranyl acetate and Reynold's lead citrate. Additionally, polysaccharides were stained using the periodic acid–silver methenamine method (Thiéry and Rambourg, 1967) in 2% (w/v) thiocarbohydrazide for 48 h. Ultra-thin sections were observed under a Phillips EM 410 transmission electron microscope (Eindhoven, The Netherlands) at 60 kV. Bacteria expressing fluorescent proteins were inoculated onto plants grown in vermiculite and observed with a 510 Meta confocal microscope (Zeiss AG, Feldbach, Switzerland). Nodule sizes were measured on a Leica MZ16 binocular microscope using Leica IM 1000 software.

Expression studies

Lotus roots were collected 4 wpi and immediately frozen in liquid nitrogen. Total RNA was extracted using the 'Plant RNeasy' kit from Qiagen AG (Hombrechtikon,

Switzerland), according to the manufacturer's recommendations. Use of the 'RLC' solution for cell lysis greatly improved quality and quantity of RNA extracted. The presence of contaminating DNA was checked using PCR primers targeting the untranscribed *NIN* gene promoter (Radutoiu *et al.*, 2003). When a single DNase I (Promega, Madison, WI, USA) treatment was not sufficient to remove traces of DNA completely, a second similar treatment was performed. The first strand was reverse transcribed using a Bio-Rad I-Script Select kit (Bio-Rad Laboratories, Hercules, CA, USA) prior to quantitative PCR performed on an I-cycler thermocycler (Bio-Rad) using a Bio-Rad SYBR Green kit (IQ-SYBR Green Supermix) following the manufacturer's instructions. Each experiment was independently performed three times.

Acetylene reduction activity

Between 2 mg and 10 mg fresh weight (along with 2 mm of root on either side) of 4 wpi nodules were incubated with 100 µl of acetylene for 2 h in a 2 ml flask at room temperature and analysed with a Dani 8521 gas chromatograph equipped with a Porapak T column (Dani, Monza, Italy) using standard protocols (Williams and Broughton, 1979).

Results

Nodulation and growth of Lotus *with NGR234 or MAFF303099*

Eleven *L. japonicus* ecotypes were inoculated with NGR234 or MAFF303099 and assessed 4 wpi. All ecotypes formed various numbers of red and green nodules with both micro-symbionts. Despite the fact that some ecotypes formed more red nodules with NGR234 than with MAFF303099 (e.g. MG-2), all grew better when inoculated with MAFF303099 (Table 1). Plants inoculated with NGR234 remained small, with yellow leaves, and were similar in appearance to the mock-inoculated controls. In contrast, those inoculated with MAFF303099 were 1–2 cm taller with dark green leaves (data not shown).

L. japonicus cv. MG-20 (hereafter MG-20) was used in all further experiments. MG-20 forms red (indicative of functional) nodules with NGR234, but the interaction yields visible rewards for the plant only from 6 to 8 wpi. This contrasts dramatically with plants inoculated with MAFF303099, where the interaction is more efficient and differences in plant size, relative to mock-inoculated controls, are visible from 3 to 4 wpi. This enhanced growth continues over time, to the point when MG-20 inoculated with NGR234 had developed to be significantly larger and greener than the control; plants grown in the presence of MAFF303099 were ~10 times larger and seeding (Fig. 1).

Nodule formation

At 4 wpi, MG-20 plants inoculated with NGR234 formed slightly fewer nodules relative to plants inoculated with MAFF303099. A significant proportion of these nodules were green (presumably ineffective) however (Fig. 2A). To determine whether there were any obvious differences in infection thread development, both rhizobial strains were labelled with green fluorescent protein (GFP). Classic

Table 1. Nodulation tests with 11 ecotypes of *L. japonicus* and *Rhizobium* sp. NGR234 or *M. loti* MAFF303099 as micro-symbionts

Plants were harvested 4 weeks post-inoculation (wpi). Numbers represent the total nodule number and stem length per pot, i.e. for three plants, as averages and standard deviations (four pots per test).

Lotus ecotypes	Strains	Green nodules	Red nodules	Total nodules	Stem length(mm)
MG-2	MAFF303099	5 ± 3	3 ± 2	8 ± 3	63 ± 18
	NGR234	9 ± 2	8 ± 3	17 ± 3	40 ± 7
MG-5	MAFF303099	10 ± 2	4 ± 3	14 ± 2	47 ± 10
	NGR234	8 ± 2	1 ± 1	9 ± 1	22 ± 3
MG-8	MAFF303099	4 ± 2	4 ± 2	8 ± 4	72 ± 15
	NGR234	8 ± 3	6 ± 3	14 ± 5	38 ± 8
MG-12	MAFF303099	3 ± 2	6 ± 2	8 ± 2	57 ± 6
	NGR234	22 ± 5	8 ± 1	30 ± 6	33 ± 7
MG-20	MAFF303099	0 ± 0	6 ± 1	6 ± 1	75 ± 10
	NGR234	1 ± 1	3 ± 1	5 ± 1	50 ± 11
MG-23	MAFF303099	1 ± 1	8 ± 2	8 ± 2	62 ± 13
	NGR234	4 ± 1	2 ± 1	6 ± 1	31 ± 8
MG-39	MAFF303099	1 ± 1	5 ± 3	5 ± 2	97 ± 19
	NGR234	9 ± 2	4 ± 2	13 ± 4	65 ± 14
MG-40	MAFF303099	1 ± 1	4 ± 2	5 ± 2	92 ± 12
	NGR234	9 ± 5	3 ± 1	12 ± 4	49 ± 10
MG-49	MAFF303099	5 ± 3	10 ± 2	15 ± 5	70 ± 1
	NGR234	13 ± 3	11 ± 2	24 ± 4	34 ± 12
MG-52	MAFF303099	3 ± 3	6 ± 2	9 ± 2	70 ± 15
	NGR234	5 ± 2	6 ± 2	11 ± 1	31 ± 7
MG-62	MAFF303099	3 ± 3	7 ± 3	11 ± 2	69 ± 14
	NGR234	8 ± 1	7 ± 3	15 ± 2	28 ± 5

infection threads were observed with both bacteria that extended from the infection pocket of a deformed root hair towards the inner part of the root (Fig. 2B). Initiation of nodule development was also analysed at the molecular level by measuring the expression of two well studied genes induced early in nodule organogenesis; *Enod2* and the gene encoding leghaemoglobin. RNA was extracted from whole roots 4 wpi, and expression levels determined in MG-20 plants inoculated with NGR234 or MAFF303099. Both genes were expressed at similar levels in both plants (Fig. 2C).

Fig. 1. Differences in the growth of *L. japonicus* MG-20 12 weeks after inoculation with MAFF303099 or NGR234, or mock-inoculated controls.

Nodule histology and functionality

At 4 wpi, transverse sections through the central part of red nodules from plants inoculated with NGR234 revealed normal histological organization comprising a central infected zone containing enlarged infected cells together with smaller non-infected and vacuolated root cells. The most obvious difference between nodules formed by MAFF303099 and NGR234 was the presence of vacuoles in infected cells (compare Fig. 3A and B). Nevertheless, nodules containing NGR234 did not fix nitrogen as efficiently as those containing MAFF303099. Specific acetylene reduction activities of red nodules induced by NGR234 were significantly lower than those induced by MAFF303099 (Fig. 3C).

Ultrastructural analyses of red nodules from differently inoculated plants showed more important differences: in nodules containing MAFF303099, the symbiosomes intimately surrounded one or two bacteroids (Fig. 3D), whereas in nodules induced by NGR234, the symbiosomes were considerably enlarged, contained multiple (up to five or six) bacteroids, and the peribacteroid spaces were filled with fibrous material (Fig. 3E). Furthermore, large numbers of starch granules accumulated in the non-infected cells of the central zone (Fig. 3F).

Nodule maturation and breakdown

Nodules induced by MAFF303099 on MG-20 rapidly developed into spherical red organs of >1.5 mm diameter

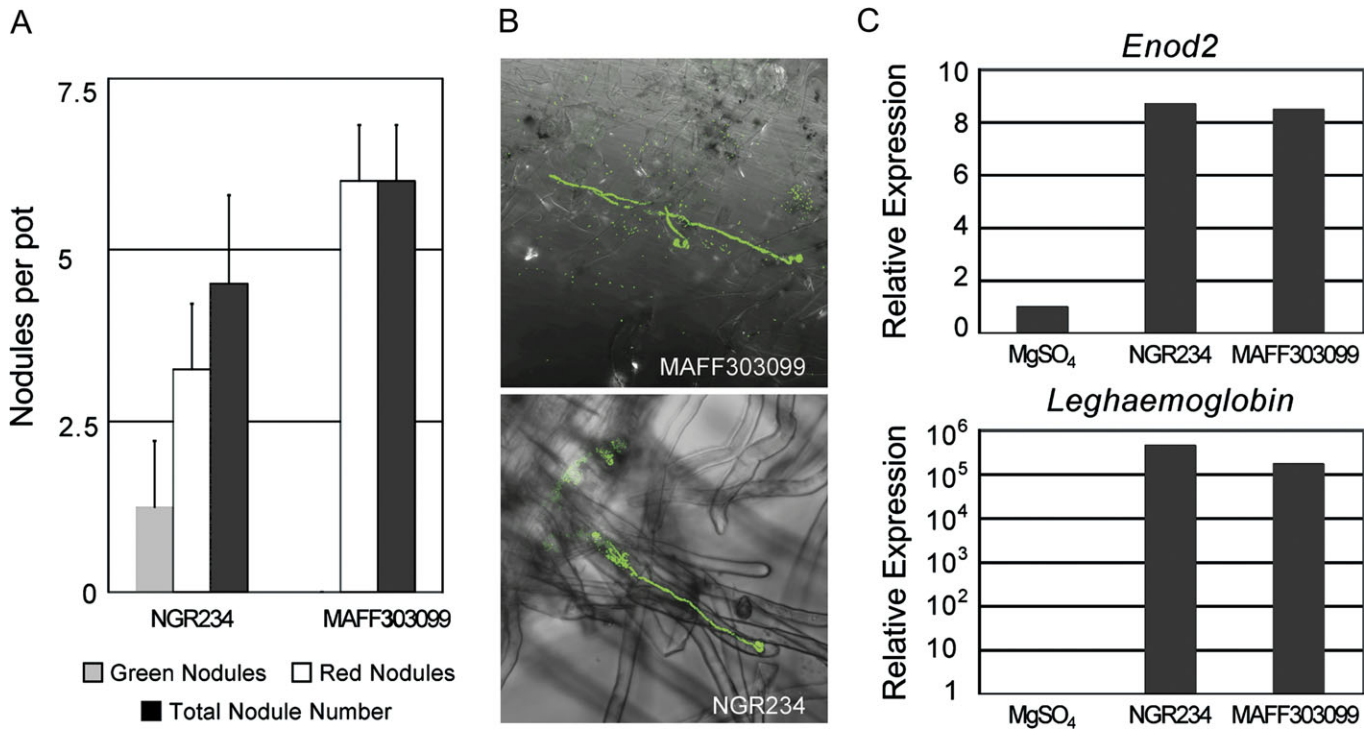

Fig. 2. Early steps of nodule development in *Lotus* inoculated with NGR234 or MAFF303099. (A) Quantification of nodules formed: nodules were counted 4 wpi. Bars represent standard deviations from the mean of four pots containing three plants each. (B) Infection thread development by MAFF303099 and NGR234 strains labelled with GFP. (C) Expression of early nodulins 4 wpi. Transcript levels were measured from complete root systems by quantitative PCR, relative to mock-inoculated (with $MgSO_4$) roots, using ATP synthase as an internal control.

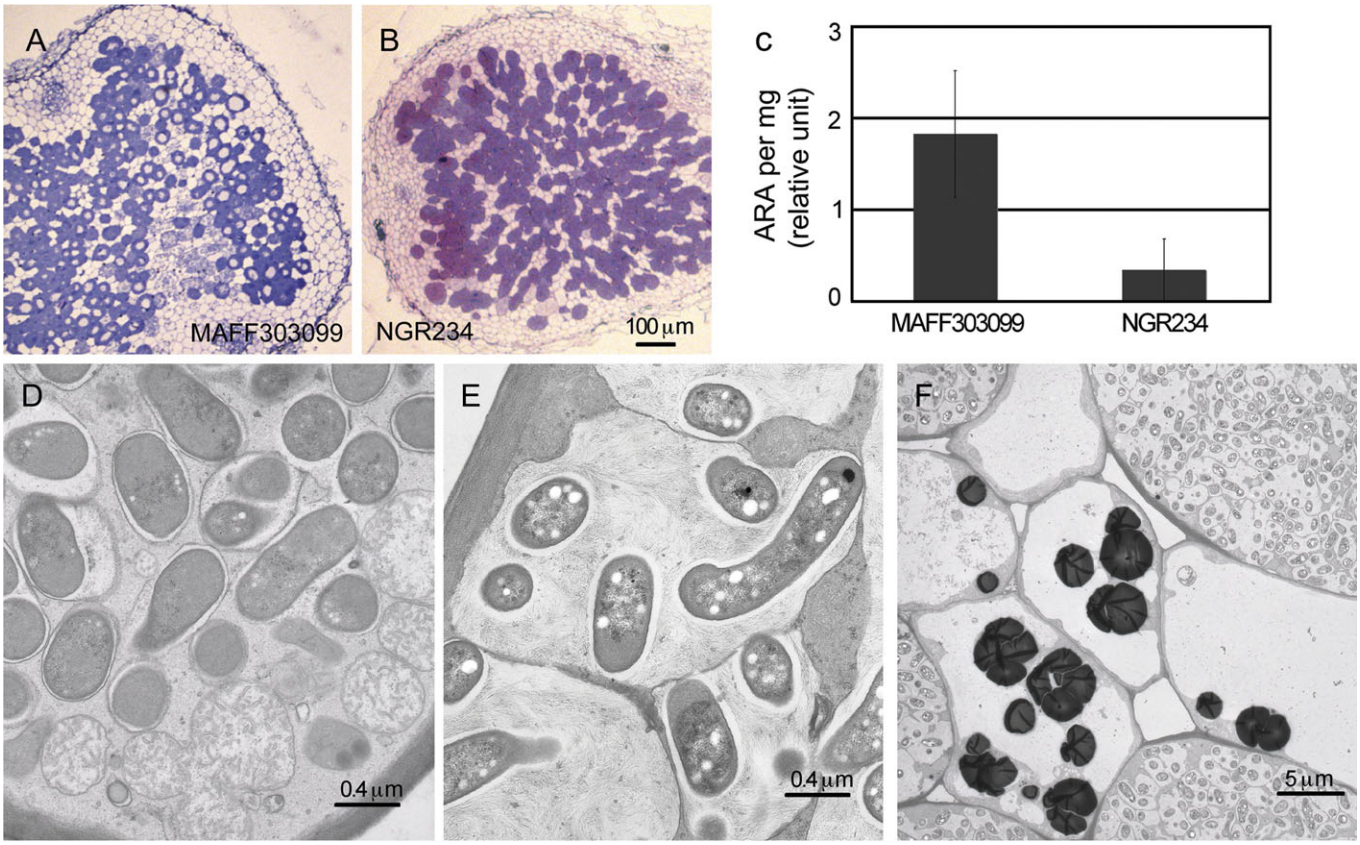

Fig. 3. Nodule morphology and functionality 4 wpi. Histological organization of transverse sections from small red nodules containing MAFF303099 (A) or NGR234 (B). Acetylene reduction activity (C); the graph shows the average of four measurements made on total nodules collected from four different pots containing three plants each; error bars represent standard deviations. Electron micrographs of infected cells from nodules containing MAFF303099 (D) or NGR234 (E). (F) Accumulation of starch granules (stained black) observed in electron micrographs of non-infected cells from nodules containing NGR234.

(Fig. 4A, F) that were long lasting, appeared unchanged even at 20 wpi (Fig. 4A, B). At 8 wpi, when MG-20 plants inoculated with NGR234 were clearly larger than mock-inoculated controls, the majority of nodules were still <1 mm in diameter (Fig. 4C, 4E). At 20 wpi, NGR234-inoculated plants were even larger (data not shown) and possessed red nodules, a few of which had diameters of >3 mm (Fig. 4D).

Biliverdin, a green catabolic product of (leg)haemoglobin metabolism, is a useful indicator of nodule senescence. Under the growth conditions used here, some *L. japonicus* ecotypes inoculated with MAFF303099 formed green nodules (Table 1), but this was very rare in the MG-20–MAFF303099 interaction, even after extended periods. Green nodules were frequently observed after NGR234 inoculation, but were always small (<1 mm in diameter). These nodules were compared with those induced by *R. etli* CE3 that undergo premature senescence (Banba *et al.*, 2001). By 4 wpi, the majority of MG-20–CE3 nodules had become necrotic with resulting dark brown coloration (Supplementary Fig. S1 at *JXB* online), a condition not seen in the MG-20–NGR234 combination. It is therefore suggested that the small green nodules sometimes seen on MG-20 inoculated with NGR234 are the consequence of early incompatibility in nodule formation, rather than senescence of fully formed nodules.

Dual symbiosome structures within cells infected with NGR234

Examination of 79 infected cells taken from different red nodules 4 wpi revealed zones with different symbiosome morphology. Although enlarged symbiosomes were predominant, occasional small sectors containing symbiosomes with only one or two bacteroids were also seen (Fig. 5A). These symbiosomes were virtually indistinguishable from those containing MAFF303099 (Fig. 3D). This type of symbiosome was termed 'tight'. Eight wpi, when MG-20 plants inoculated with NGR234 were visibly larger than controls, these sectors containing tight symbiosomes had increased in size and were more frequent (Fig. 5B). Furthermore, individual tight symbiosomes were found mixed with enlarged symbiosomes in approximately equal proportions (Fig. 5C).

In even older nodules, 7 months after inoculation with NGR234, sectors of tight symbiosomes were no longer visible. Some infected cells contained totally tight symbiosomes, while others showed both tight and enlarged structures (Fig. 6A, B). Occasionally infected cells

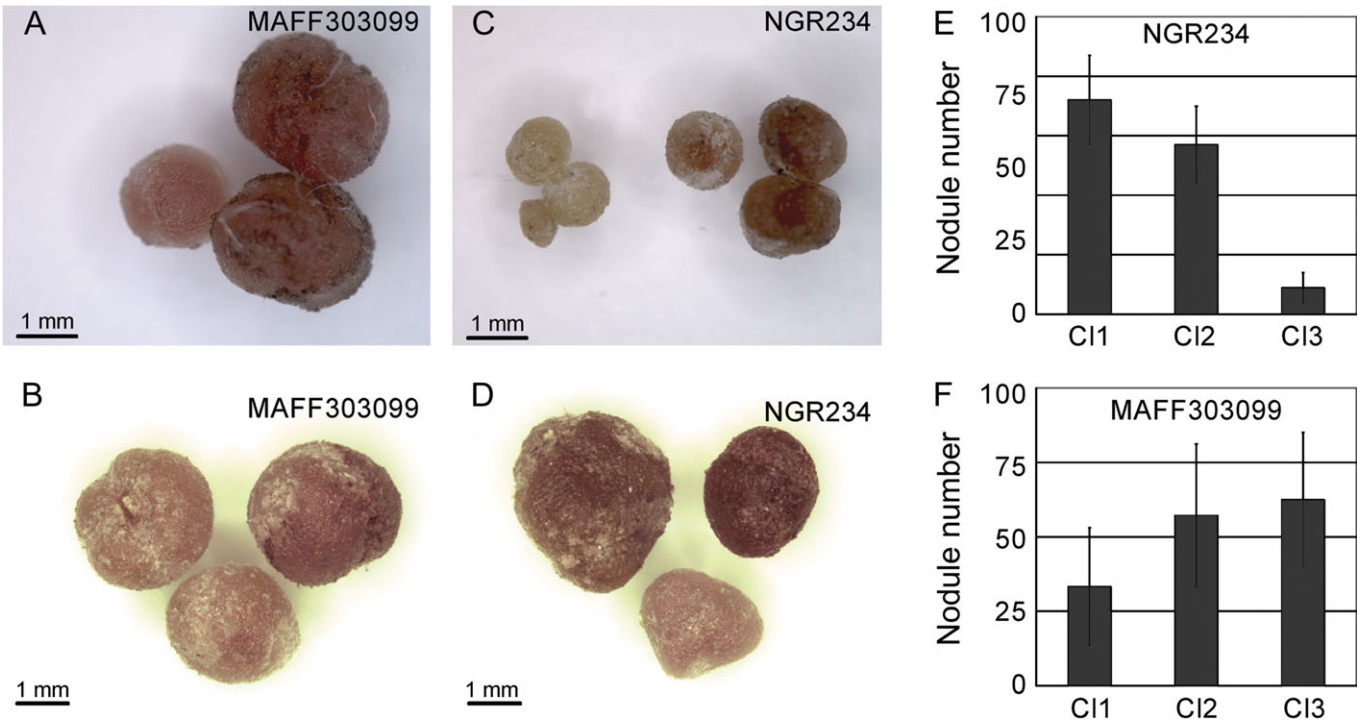

Fig. 4. Nodule appearance (size and colour) at later stages of the symbiotic interaction. MG-20 nodules induced by NGR234 and *M. loti* 8 wpi (A & C) or 20 wpi (B & D); MG-20 possessed exclusively red nodules (2–4 mm diameter) when inoculated with MAFF303099. In contrast, NGR234 provoked the formation of both red and small green nodules at 8 weeks (C), and at 20 weeks larger red nodules could be seen (D). The diameters of nodules were assigned to three classes (CI) at 8 wpi (CI1 <1 mm, CI2 >1 mm, and CI3 >1.5 mm). Bars represent average distributions of all the nodules from four pots with standard deviations indicated (E and F).

presented degraded contents (Fig. 6C); their appearance was similar to that of type 1 senescent cells seen in the proximal part of the fixation zone of *Medicago truncatula* (Van de Velde *et al.*, 2006).

Discussion

The term Nod^+/Fix^+ is used to describe legume–rhizobia alliances that are fully active symbiotically. If nodules fail to develop with a particular micro-symbiont, a Nod^- phenotype results, and if nodules develop but do not fix nitrogen the interaction is called Nod^+/Fix^- (Pueppke and Broughton, 1999). Neither of these latter two phenotypes is able to sustain plant growth under nitrogen-deficient conditions. In this sense, the *L. japonicus*–NGR234 interaction falls in the Nod^+/Fix^+ category along with the symbiosis between *L. japonicus* and its natural micro-symbiont *M. loti*. Nodules induced by NGR234 develop in a classical manner, infection threads that penetrate root hairs were formed, and levels of nodulin transcripts in *L. japonicus* nodules were comparable with those found using *M. loti* as the inoculum. Inoculation by NGR234 or MAFF303099 resulted in similar nodule numbers 4 wpi and transverse sections showed normal structure. Furthermore, nodules containing NGR234 fixed nitrogen, were long-lasting (Fig. 6), and, after several months, could be as large as mature nodules containing MAFF303099 (Fig. 4). Thus despite not being the regular micro-symbiont of *L. japonicus*, the interaction with NGR234 is stable and clearly different to that reported with *R. etli* CE3 (Banba *et al.*, 2001). NGR234 contributes to plant growth under nitrate-limiting conditions and normal nodule functions do not collapse.

In comparison with nodules induced on *L. japonicus* by MAFF303099, those containing NGR234 are only moderately efficient, however, and present clear differences in their ultrastructural organization. Four wpi, almost all symbiosomes were enlarged, and similar in appearance to those induced by nitrogenase-deficient strains (Hahn and Studer, 1986; Regensburger *et al.*, 1986) or to those formed by plant mutants of the Nod^+/Fix^- category (Kumagai *et al.*, 2007). Enlarged, starch-containing symbiosomes are frequent in inefficient nodules (Hirsch *et al.*, 1983; Niehaus *et al.*, 1993; Barsch *et al.*, 2006) and are indicative of poor nitrogen fixation. A thorough examination of nodule sections (4 wpi) revealed rare sectors containing bacteroids tightly enclosed in peribacteroid membranes. The appearance of such sectors was not observed in interactions between *L. japonicus* and the other (alternative) micro-symbionts *R. etli* or *R. tropici*. In these interactions, occasionally 'tight' individual symbiosomes were seen, but the vast majority were enlarged (data not shown). As the NGR234-containing nodules matured, enlarged symbiosomes became less predominant, however: tightly enclosed bacteroids dominated, with some infected cells containing only tight symbiosomes. In the development of

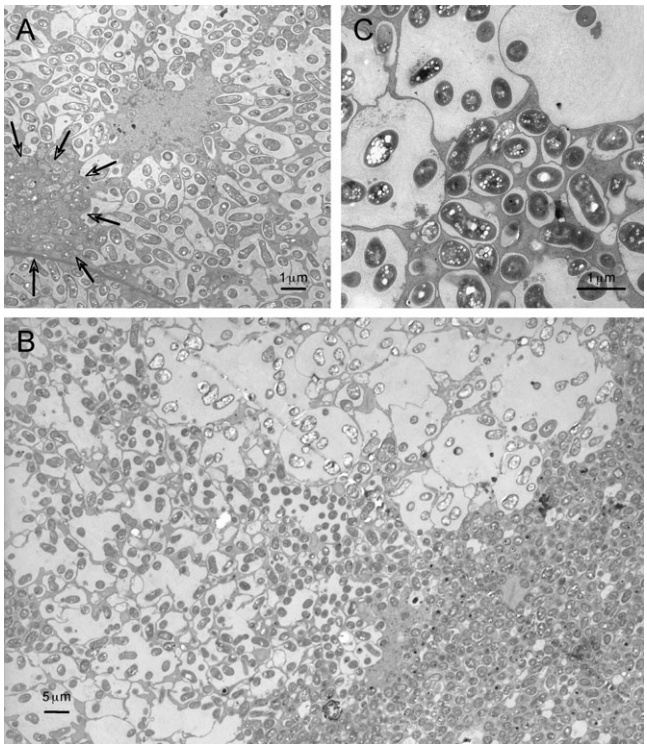

Fig. 5. Electron micrographs of infected cells in nodules containing NGR234. (A) At 4 wpi, small sectors of tight symbiosomes were confined to restricted rare zones of the infected cells (delineated by arrows). By 8 wpi, such sectors were larger and more frequent, sometimes occupying most of the infected cell (B). Bacteroids tightly enveloped in a peribacteroid membrane could also be found intermingled with enlarged symbiosomes in 8 wpi nodules (C).

determinate nodules, such as those found on *L. japonicus*, there are two stages; first when the infected cells continue to divide and then later when they enlarge. After rhizobia enter the host cells, the infected cells stop dividing and undergo several cycles of endoreduplication, which result in increased cell growth (Truchet *et al.*, 1980; Bergersen, 1982; Brewin, 1991). Simultaneously, plants direct the differentiation of rhizobia into bacteroids (Mergaert *et al.*, 2006). An investigation was carried out to determine whether there could be a correlation with this switch from host cell division to enlargement and the appearance or increase in the numbers of tight symbiosomes.

The morphology of symbiosomes from large, extremely mature (7-month-old) nodules where infection events should have stopped argues against this, however. Although some of the infected cells contained only tight symbiosomes, infected cells still containing enlarged symbiosomes interlaced with tight ones were found within the same nodule, in approximately equal numbers (Fig. 6). Thus the correlation between cell enlargement and the formation of tight symbiosomes does not completely parallel maturation of the infected cell.

Tight symbiosomes and the nodule sectors containing them are considered to be more active in nitrogen fixation, and the increase in their relative proportion with age correlates with the increased contribution of NGR234 to the growth of *L. japonicus*. In other words, the initial very loose association between bacteroid and symbiosome membranes, frequently observed in Nod^+/Fix^- interactions (Hahn and Studer, 1986; Regensburger *et al.*, 1986; Kumagai *et al.*, 2007), does not compromise *per se* the potential of MG-20–NGR234 to fix nitrogen as the nodules age. It also suggests that the minimal fixed nitrogen supply from these small sectors of active symbiosomes is sufficient to prevent nodule senescence and sustain the development of the whole organ. Thus a critical level of symbiotic activity apparently avoids plant-triggered nodule degradation and subsequently leads to the development of more efficient nodules. For as yet unknown reasons, there is a delay in the ability of NGR234-containing nodules to reach this level of nitrogen fixation. Thus, plant growth is also delayed.

Legume growth in nitrate-limiting conditions directly reflects the ability of rhizobia to reduce atmospheric nitrogen to ammonia and thus requires an effective symbiotic association. In the field, symbiotic efficiency is not dependent on interstrain competitiveness, i.e. the ability to develop new fixing organs (Amarger, 1981; Hahn and Studer, 1986), and competitive strains are often poorly efficient (Thies *et al.*, 1991; Sadowsky and Graham, 1999). Nitrogen conversion efficiency also varies with legume–rhizobia combinations and environmental conditions (Vlassak and Vanderleyden, 1997; Zahran, 1999). These factors mostly alter nodule functioning as opposed to nodule development. Nevertheless, most published data related to the legume–rhizobial symbiosis are focused on nodule initiation/development rather than nodule functionality. Mutations involved in yield modification are often overlooked during genetic screens in comparison with phenotypes showing more dramatic symbiotic phenotypes, e.g. Nod^- or Fix^- mutants. As a consequence, the mechanisms by which rhizobia establish long-term (nitrogen-fixing) interactions and persist within cells of the legume host remain largely unknown. We suspect that the ineffectiveness of the *L. japonicus*–NGR234 symbiosis is due to a delay in the establishment of the persistent stage of cell infection. For intracellular pathogens, persistent (or chronic) infection is a phase distinct from that leading to cell invasion and requires specific gene sets (Rhen *et al.*, 2003). It is possible that rhizobia utilize similar molecular mechanisms, and that after nodule initiation there is a continuation of the molecular dialogue between the symbionts after rhizobial internalization. For *L. japonicus* it seems that NGR234 has sufficient signals to permit tolerance, whereas *R. etli* CE3 lacks or has different persistence mechanisms.

In conclusion, the interaction between *L. japonicus* and NGR234 presents all the hallmarks of a typical stable, legume–rhizobia symbiosis and, for this reason, it is possible to study nodule initiation taking advantage of the well-characterized micro-symbiont NGR234 (Perret *et al.*, 2000). Furthermore the nature of this specific interaction will permit the investigation of rhizobial persistence and

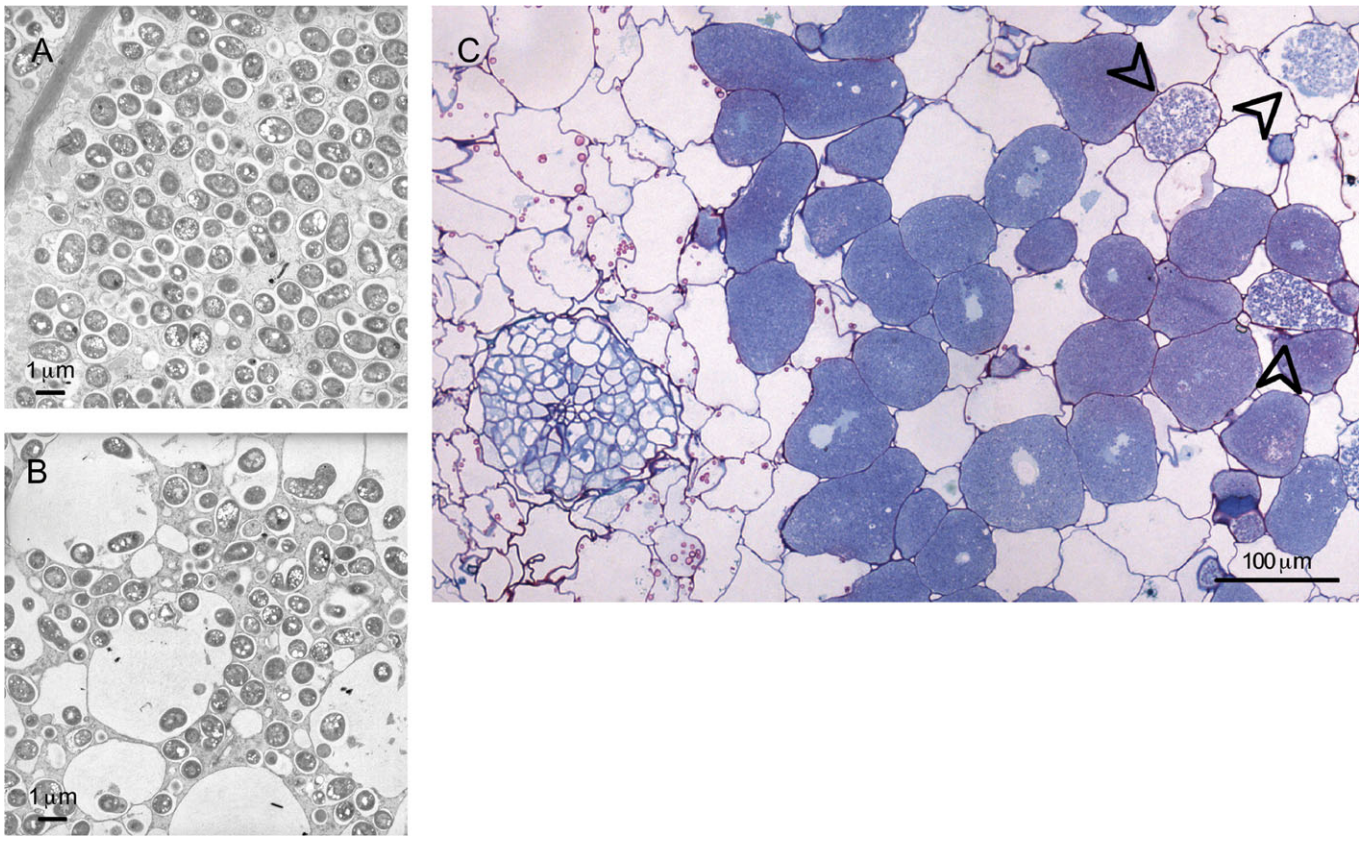

Fig. 6. Electron (A & B) and light (C) micrographs of 7 month-old nodules containing NGR234. Infected cells contained predominantly bacteroids closely-enveloped within peribacteroid membranes (A), or a mixture of both tight and enlarged symbiosomes (B). Viewing at lower magnification revealed some of the infected cells, dispersed throughout the nodule, presenting degraded content (C; labelled with arrowheads).

plant tolerance strategies within *L. japonicus* nodules. These are essential aspects of legume–rhizobia interactions often overlooked in classical forward genetic screens.

Supplementary data

Bacterial strains and plasmids used in this work, as well as necrotic nodules observed after inoculation of *L. japonicus* cv. MG-20 with *Rhizobium etli* CE3, are presented in Supplementary Table S1 and Supplementary Fig. S1, respectively, available at *JXB* online.

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